The therapeutic effects of cyclosporin-A on experimental spinal cord injury.

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Abstract

Background: According to the experiments, neutrophils and microglial cells are the first to attend the early phase of events in inflammatory response to SCI. Those pilot cells are seen in the first 12-24 hours and disappear about 3-5 days. The neutrophil accumulation and activation are steered by many cytokines such as TNF-α, IL-1 and IL-6. Neutrophils do accompany to the modulation of secondary injury mechanisms via neutrophil proteases and reactive oxygen molecules. When those processes are taken into account, depletion of neutrophils or depression of their functions may derive neuro-protection and neurological healing.

Purpose: To investigate the therapeutic and neuroprotective effects of Cyclosporin-A (CSA) on recovery processes using clinical and histopathological tests, which has not been used very frequently in spinal compression cord injury (SCI) models.

Material and methods: Twenty-four Spraque-Dawley rats were divided into three groups: group 1 [Sham-control, n=8], group 2 [SCI+2 mL saline intramuscular (i.m.), n=8], group 3 [SCI+5 mg/kg CSA (i.p.) 1 h after SCI and for the following three days, n=8]. Rats were evaluated 1st, 3rd, 5th and 10th days after SCI, clinically by Drummond and Moore scale and under light microscopy and by TUNEL test; after scarification on 10th day.

Results: Clinical and histopathological results of treatment group were found significantly better than the results of the trauma group.

Conclusion: CSA can depress apoptosis and necrosis rates in a statistically significant manner and carry out the statistical difference in clinical results.

Keywords: Animals, Cyclosporine, Immunosuppressive agents, Motor activity, Spinal cord injuries, Thoracic vertebrae.

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Introduction

First reaction of body to injury and infections is particularly the inflammation. The role of inflammation after spinal cord injury (SCI) is defined in details; but profitable and destructive effects are still debatable. In general aspect, although early stage inflammatory events are not welcomed in neurotrauma, they are thought to be in favour on late stages [1]. According to the experiments on animals and humans, neutrophils and microglial cells are the first to attend the early phase of events in inflammatory response to SCI [2-5]. Those pilot cells are seen in the first 12-24 hours and disappear about in 3-5 days [1]. The neutrophil accumulation and activation are steered by many cytokines such as TNF-α, IL-1 and IL-6 [6]. Neutrophils do accompany to the modulation of secondary injury mechanisms via neutrophil proteases and reactive oxygen molecules [7]. Minutes or even hours after SCI, those cells are activated or transform into macrophages. Macrophages add more to the destructive effects by releasing pro-inflammatory cytokines, reactive oxygen radicals, nitrous oxide and proteases [8]. They lead many biological substrates to change in a pathological manner, such as peroxidation of the lipid components of the oxidative stress cells. The results of an early staged inflammation; like ischemia, cell/tissue edema, oxidative degradation, myelin degradation, necrosis and apoptotic changes, may increase the volume of the lesion [9]. Furthermore, those changes give rise to glial scar tissue and development of the infection protective environment; thence hinder creation of a successful regeneration [10]. When those processes are taken into account, depletion of neutrophils or depression of their functions may derive neuro-protection and neurological healing [11].

This study aims to investigate the protective (neuroprotective) and therapeutic (regenerative) effects of the cyclosporin-A, which is an immunosuppressive drug, on secondary spinal cord injury in a rat spinal injury model; in structural and also functional aspects.
Material and Methods

Experimental groups

The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and approval was received from the Institutional Animal Ethics Committee at Abant Izzet Baysal University. Twenty four female and adult Sprague Dawley rats, weighing 250-300 g, were divided randomly into three groups; composed of eight rats in each:

Group 1: Control-Sham (only laminectomy, no SCI, n=8)
Group 2: Trauma (SCI, 2 mL saline intraperitoneally [i.p], n=8)
Group 3: Trauma+CSA (SCI, single 5 mg/kg cyclosporin-A [Sandimmun Novartis] injected i.p. immediately after SCI and for the following three days, once a day, n=8)

All rats were evaluated on 1st, 3rd, 5th and 10th days after SCI, clinically by Drummond and Moore scale, under light microscopy and by TUNEL test; after sacrifice on day 10.

Surgical procedure

The rats were anesthetized with intramuscular injection of 10 mg/kg xylazine (Bayer, Istanbul, Turkey) and 60 mg/kg ketamine hydrochloride (Parke Davis, Istanbul, Turkey) before surgery. With the rats in a prone position, a T6-T10 midline incision was made. Three-level laminectomies (T7-T9) were performed, leaving the dura matter intact, and SCI was produced by extradural compression of the spinal cord using an aneurysm clip with a closing force of 24 g. In all of the injured groups, the spinal cord was compressed for 1 min. Sham-injured animals were only subjected to laminectomy. After surgery, 2.0 cc of saline was administered intraperitoneally to the rat ad libitum. During this time period, the bladders of the animals were manually voided twice a day until the mice were able to regain normal bladder function. At the end of day 10, all rats were killed under deep anaesthesia.

Approximately 10 mm of spinal cord from the level between T7 and T9 was obtained from each rat, and in the trauma groups the cord sample was divided 3 mm below the epicenter of the injury. The samples from the lower levels of the spinal cord lesion (epicenter) were used for histological and immunohistological analyses.

Neurological evaluation

Grading of motor disturbance: The hind limb motor function of rats were evaluated once a day on days 1, 3, 5 and 10 after SCI by an independent observer according to the Drummond and Moore scale [12]. A score of 0 to 4 was assigned to each animal as follows: 0=paraplegic with no evident lower extremity motor function; 1=poor lower extremity motor function, flicker of movement, weak antigravity movement only; 2=moderate lower extremity function with good antigravity strength but inability to draw legs under body and/or hop; 3=the ability to draw legs under body and hop, but not normally; 4=normal motor function.

Histological evaluation

Light microscopy: Spinal cord biopsies were taken on 10th day. The biopsies were fixed for 24 h in paraformaldehyde solution (4% in 0.1 M PBS) at room temperature, dehydrated by graded ethanol, and embedded in paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (thickness 5 μm) were deparaffinised with xylene, stained with H&E and studied using light microscopy (Dialux 22 Leitz; DBA srl). All the histological studies were performed in a blinded fashion. Extensity of the necrosis was evaluated in three main specimens which were collected 3 mm caudal to the epicenter and specimens were analyzed for 4 criteria established for acute spinal cord injury by Black et al. [13], as follows: 1-white matter degeneration, characterized by edema, formation of cysts, demyelination and infiltration of macrophages, cystic necrosis and cytoarchitectonic disorganization. 2-haemorrhage in white or gray matter; 3-neuronal loss, sometimes with vacuolization and inflammatory infiltration in gray matter; 4-signs of hypoxic injury; nuclear retraction and pyknosis, as well as intense cosinophilic staining of the pericardium. Based on the described criteria, histological alterations related to the intensity of necrosis were classified semi-quantitatively into the following four categories after scanning of all slices; <1% of total scanned area: score 0; 1-24% of total area: score 1; 25-49%: score 2; 50-74%: 3; and finally >75%: score 4. This quantification was performed by one independent and experienced pathologist on three different sections in a blinded manner and without knowledge of the experimental group.

Terminal Deoxynucleotidyltransferase-Mediated UTP end labelling assay (TUNEL)

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer’s instruction (Apotag, HRP kit; DBA srl). In brief, sections were incubated with 15 g/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H2O2 for 5 min at room temperature and then washed with PBS. Sections were immersed in TdT buffer containing deoxynucleotidyl transferase and biotinylated UTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine. The site of the trauma on the spinal cord was accepted as the "epicenter" and apoptotic cells in three different main sections which were collected 3mm caudal to the epicenter, were counted.
**Statistical analysis**

SPSS (Statistical Package for Social Sciences) for Windows 10.0 was used in the analysis. Since parameters had no regular distribution, Kruskal Wallis test was used in comparisons of quantitative data from groups. And Mann-Whitney U test was preferred to detect the group which causes the difference. When comparing the parameters within the groups, Wilcoxon signed rank test was used. Results were evaluated in a 95% confidence interval and the significance was accepted at the level of p<0.05.

**Results**

Reduction in the body weights of the subjects before and after the experiment was significant. After day 7, significant atrophy in the lower extremity muscles was observed in the trauma group whereas it was not prominent in the treatment group. After the sacrifice of the subjects, macroscopic edema and hemorrhage was seen on extracted medulla spinalis structures.

**Neurological results**

Grading of motor disturbance: On days 1, 3, 5 and 10 the difference regarding the motor function scores between all groups remains significant (p<0.05) (Table 1). When binary comparisons of the groups were evaluated it has been seen that in duration of the whole test, group 2 (trauma) and group 3 (CSA) were not able to reach the scores of the SHAM group regarding the motor scores (p<0.05). But starting from day 5, a significant difference was observed in favour of group 3 when group 2 was compared with group 3 (p:0.002) and this difference got more prominent on day 10 (p:0.001) (Table 2 and Graph 1). According to the in-group analysis of the groups with Wilcoxon test, only group 3 (CSA) had significant difference regarding the day 1 and 10 comparisons (Table 3).

**Table 1.** Comparison of the three groups on 1st, 3rd, 5th and 10th days regarding the Motor Function Scores, by Kruskal-Wallis test (p<0.05).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham mean</th>
<th>Sham St error</th>
<th>Trauma mean</th>
<th>Trauma St error</th>
<th>Cyclosporin-A mean</th>
<th>Cyclosporin-A St error</th>
<th>Comparison of the groups by Kruskal-Wallis test p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-1</td>
<td>3.83</td>
<td>0.167</td>
<td>0.33</td>
<td>0.211</td>
<td>0.33</td>
<td>0.211</td>
<td>0.002</td>
</tr>
<tr>
<td>MS-3</td>
<td>3.83</td>
<td>0.167</td>
<td>0.33</td>
<td>0.211</td>
<td>1.17</td>
<td>0.307</td>
<td>0.001</td>
</tr>
<tr>
<td>MS-5</td>
<td>3.83</td>
<td>0.167</td>
<td>0.33</td>
<td>0.211</td>
<td>1.83</td>
<td>0.167</td>
<td>0.000</td>
</tr>
<tr>
<td>MS-10</td>
<td>4</td>
<td>0</td>
<td>0.33</td>
<td>0.211</td>
<td>2.83</td>
<td>0.167</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Table 2.** In-group comparison of the Motor Score data by Wilcoxon test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Motor score 1</th>
<th>Motor score 3</th>
<th>Motor score 5</th>
<th>Motor score 10</th>
<th>Necrosis</th>
<th>Necrosis cells</th>
<th>Necrosis apoptotic</th>
</tr>
</thead>
<tbody>
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<td>Groups 1, 2</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Groups 1, 3</td>
<td>0.002</td>
<td>0.003</td>
<td>0.002</td>
<td>0.001</td>
<td>0.002</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Groups 2, 3</td>
<td>1</td>
<td>0.057</td>
<td>0.004</td>
<td>0.002</td>
<td>0.014</td>
<td>0.008</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** In-group comparison of the Motor Score data by Wilcoxon test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Motor Score 3 - Motor Score 1</th>
<th>Motor Score 5 - Motor Score 1</th>
<th>Motor Score 10 - Motor Score 1</th>
<th>Motor Score 5 - Motor Score 3</th>
<th>Motor Score 10 - Motor Score 3</th>
<th>Motor Score 5 - Motor Score 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM Z</td>
<td>0.000b</td>
<td>-1.000c</td>
<td>-1.000c</td>
<td>-1.000c</td>
<td>-1.000c</td>
<td>-1.000c</td>
</tr>
<tr>
<td>SHAM Asymp. Sig.</td>
<td>1.000</td>
<td>0.317</td>
<td>1.000</td>
<td>0.317</td>
<td>1.000</td>
<td>0.317</td>
</tr>
<tr>
<td>Cord injury Z</td>
<td>0.000b</td>
<td>0.000b</td>
<td>0.000b</td>
<td>0.000b</td>
<td>0.000b</td>
<td>0.000b</td>
</tr>
<tr>
<td>Cord injury Asymp.</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Cord injury+Cyclosporin-A Z</td>
<td>-2.236c</td>
<td>-2.251c</td>
<td>-2.000c</td>
<td>-2.271c</td>
<td>-2.449c</td>
<td>-2.000c</td>
</tr>
<tr>
<td>Cord injury+Cyclosporin-A Asymp.</td>
<td>0.25</td>
<td>0.024</td>
<td>0.046</td>
<td>0.023</td>
<td>0.014</td>
<td></td>
</tr>
</tbody>
</table>
Graph 1. Comparison of the five groups on 1st, 3rd, 5th and 10th days regarding the Motor Function Scores, by Kruskal-Wallis test.

Histological results

Light microscopy results: According to the Hemotoxylin-Eosin dye staining necrosis results on day 10, there was a significant difference between all groups (p: 0.00; p<0.01) (Table 2). It was seen that there is a high level of significant difference in binary comparison of the groups according to the necrosis scores, between Group 1 (control-Sham) and other groups (Figure 1). When Group 2 was compared with group 3 (CSA) it was noted that groups had statistically significance difference, in favor of treatment group (p: 0.008) (Table-2; Graph-2). Briefly cyclosporin-A causes a statistical difference in prevention of necrosis when compared with the trauma group.

Graph 2. Comparison of the groups regarding Necrosis and Apoptotic cell numbers.

Figure 1. a: A section from the Sham group. Hematoxilen-Eosin. b: An image from the spinal cord injury group. Loss of tissue integrity, edema (*) and increase in number of inflammatory cells. (i) is seen on the lesion site-core area. Hematoxilen-Eosin. C: A section from the Cyclosporin-A group after the spinal cord injury. It can be seen that lesion site-core area is more limited (→). Hematoxilen-Eosin.
**Terminal deoxynucleotidyltransferase-Mediated UTP end labeling assay (TUNEL) results**

Outcomes of TUNEL on day 10 pointed out that there was a statistical difference in between all groups (Table 2). On day 10 comparing the number apoptosis in binary groups, a significant difference between Group 1 and other groups was seen (Figure 2). Also when group 2 (trauma) and group 3 (CSA) are compared a significant statistical difference in favor of therapy group is seen (p: 0.008) (Table 2 and Graph 2). In preventing the apoptosis Cyclosporin-A has a significant difference when compared with the trauma group.

![Image](https://via.placeholder.com/150)

**Figure 2.** a: Apoptotic cells on a section from the Sham group (→). TUNEL b: Increased number of apoptotic cells on a section from spinal cord injury group can be seen (→) TUNEL. c: On a section from Cyclosporin-A group it can be seen that the number of apoptotic cells are fewer than the injury group (→) TUNEL.

**Discussion**

Immunosuppressive agents can be divided into four major groups: 1. T-cell blockers (Cyclosporin-A, Tacrolimus (FK506), Sirolimus), 2. Glucorticoids, 3. Cytotoxic drugs, 4. Anticore Markers (reagent). As a T-cell blocker, Cyclosporin-A is the most frequently and effectively used agent as an immunosuppressive drug. Cyclosporin-A is a cyclicolipopeptide, composed of 11 amino acids and derived from a fungus named Tolypocladiuminflatum Gams. Following the recognition of the antigen, cyclosporin-A creates immunosuppression by preventing the early stages of the autoimmune response. It cannot treat an autoimmune response, which occurred previously, or an occurred rejection reaction; but it may prevent a reponse or reaction if it is given prior to occurrence. It selectivly inhibits inductor/helper subtype T lymphocytes (CD4+) and blocks their proliferation and differentiation [14]. It has no effect on the mature T cells. So it should be used as soon as possible after the encounter with an antigen (for example in an allogenictransplantation), preferentially in the first 24 hours [14]. Therefore we have adminstered the treatment right after the trauma. It has its immunosuppressive and secondary neuronal injury preventive effect on CD4+ T lymphocytes by inhibiting calcineurin, which is a Ca++ depended enzyme and one of the steps of the signalling cascade that is initiated by stimulation of the T lymphocyte receptor with antigen. In this chain of reactions, cyclosporin-A inhibits Ca++ transportation via mitochondria membrane trasportation (MMT) which is located on the mitochondria membrane. In order to have this effect cyclosporin-A should bind to cyclophilin, which is a cytoplasmic receptor. Binding of cyclosporin-A to cyclophilin causes a protein complex which inhibits activation of calcium/calmodulin dependent calcineurin.This complex inhibits calcineurin or protein phosphatase 2B [14]. If this calcineurin is not inhibited it causes dephosphorilation of nuclear factor of activated T-cells.
(NTF) which regulates the release of cytokines like IL-2, IL-3, GM-CSF (Granulocyte colony stimulating factor) and TNF-α [15]. Main clinical effect of cyclosporin-A is probably the inhibition of IL-2 which is produced by T-cells; that which is inhibiting IL-2 that induces proliferation of other T-cells.

An important stage of inflammation after neurul trauma is the activation of microglia and leucocyte infiltration [16]. Microglial cells are known to be the immune cells of the central nervous system (CNS). After infiltration of the ischemic tissue by microglia and astrocytes, cytokines (IL-2, IL-3, TNF-α), which are major indicators of the inflammatory response, are released [17]. Cyclosporin-A is defined to be effective in inhibition of TNF-α and cyclooxygenase-2 expression from these microglial cells [18]. From the functional perspective, interestingly cyclosporin-A reduces the formation of NO on the microglial cell line and also probably the cytostatic mechanism is effected as well [19]. Beyond this, following the stimulation of the receptor ligands of the pheriperal benzodiazepines, production of free radicals on different microglial cell lines are blocked by cyclosporin-A [20].

Also cyclosporin-A's effect extends even through astrocytes since it is known that cyclosporin-A prominently reduces astrocytic apoptosis and the release of TNF-α, IL-1 and IL-2 from astrocytes [21,22].

Cyclosporin's effects on acute CNS disorders like cerebral ischemia was first studied on animal experimental models in the early 1990's. This pioneer studies revealeed the useful results of cyclosporin on transient ischemic forebrain injury in rats; cyclosporin was reported to reduce the infarct and edema volume [23]. But, it is very rare for cyclosporin to be used in experimental spinal cord injury and acute brain injury models; but beneficial results were reported. For example, the use of cyclosporin after complete thoracal spinal cord cuts in the rats, axonal regeneration is shown to increase and a decrease in inflammatory effects of macrophages and microglial cells was reported [24]. Same group of investigators later on reported a recovery in functional levels with cyclosporin treatment in rats with complete thoracal spinal cord cuts [25]. In studies on rats with traumatic brain injury, which investigate cyclosporin's positive effects on neurons,axonal degeneration models revealed that mitochondrial swelling, deposition of β-amiloid precursor proteins and compaction of the neurfilaments were reduced [26,27]. Cyclosporin-A application in first 6 hours, in throcal cord injury models, was shown to inhibit lipid peroxidation and enhancement in the mobility of the rats exposed to lesion [28]. On the other hand in a more recent study objected these findings, reporting no reduction in tissue loss in groups which were treated with cyclosporin and no enhancement in the motor scores [29]. But later on it has been claimed that this failure of the drug was due to insufficient dosing of the cyclosporin [30]. Most lately a study in 2010, reported that cyclosporin, which is used after transportation of oligodendrocyte precursor cells (OPC) to the traumatized spinal cord, had better histological outcomes and in correlation to this better functional recovery results were achieved by cyclosporin, when compared to the control group; but cyclosporin failed to prevent the transported oligodendrocyte precursor cells to transform into astrocytes [31]. In fluid-percussion injury models cyclosporin-A was reported to improve motor and amnestic functions in traumatic brain injury [32]. Reports, that explaining cyclosporin's effects on isolated neurons are very rare. But studies on experimentally lesioned neuroblastoma cells showed that cyclosporin prevents cells from apoptosis, inhibits caspase activation and activates neuronal growth [33,34].

Phase-II clinical researches on traumatic brain injuries are being carried out and those trials are showing the safety of cyclosporin-A. But there is a heterogenicity regarding these trials especially in timing of cyclosporin-A treatment after traumatic beyin injury [35].

There is not any clinical study in the literature about cyclosporin-A treatment in traumatic spinal cord injuries yet. And to our knowledge, no reports of clinical use of cyclosporin-A in acute CNS disorders (such as ischemia) exist. By in vitro and in vivo evaluations of these findings it is accepted that Cyclosporin-A has direct inhibitor effects on microglial cells, it has neuroprotective and neuroregenerative characteristics and cyclosporin-A has direct effects on neurons and glial cells [36].

As mentioned above there are no experimental studies in the literature, which match identically to our study. And also the number of experimental spinal cord injury studies with cyclosporin, are very limited. Palladini et al. [25] showed that groups which were treated with cyclosporin-A, had structural and functional recovery, in thoracal cord injuries. Also Diaz-Ruiz et al. [28] declared that in groups treated with cyclosporin-A after thoracal cord contussion, lipid peroxidation was decreased. But in contrast to these outcomes Rabchevsky et al. [29] reported that in both groups, treated with cyclosporin-A or not, had similar results in functional and structural recovery parameters.

According to the out-comes of the motor score recovery in both trauma groups were not significant up to day 5; whereas after day 5 in favor of "cord injury+cyclosporin group" there was a significant recovery result (Table 2). Also, when groups were analyzed with binary comparison, (Mann-Whitney U test) functional evaluation (motor score) and also structural evaluation (histologically necrosis-apoptosis) had statistically significant outcomes in favor of "cord injury+CSA" (Table 2).

Conclusion

In our experimental rat spinal cord injury model; we introduce that cyclosporin-A has neuroprotective (Necrosis/Apoptosis data in favor of cyclosporin-A group) and regenerative effects (motor score in favor of cyclosporin-A group) on secondary spinal cord injury, with statistically significant data.
The therapeutic effects of cyclosporin-A on experimental spinal cord injury.

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